

Development of Multiplex Real-Time RT-PCR as a Diagnostic Tool for Avian Influenza

Author(s): E. Spackman, D. A. Senne, L. L. Bulaga, S. Trock, and D. L. Suarez

Source: Avian Diseases, 47(s3):1087-1090. 2003.

Published By: American Association of Avian Pathologists

DOI: 10.1637/0005-2086-47.s3.1087

URL: <http://www.bioone.org/doi/full/10.1637/0005-2086-47.s3.1087>

BioOne (www.bioone.org) is an electronic aggregator of bioscience research content, and the online home to over 160 journals and books published by not-for-profit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Web site, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/page/terms_of_use.

Usage of BioOne content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

Development of Multiplex Real-Time RT-PCR as a Diagnostic Tool for Avian Influenza

E. Spackman,^A D. A. Senne,^B L. L. Bulaga,^C S. Trock,^D and D. L. Suarez^A

^ASoutheast Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Road, Athens, GA 30605

^BNational Veterinary Services Laboratory, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, P.O. Box 844, Ames, IA 50010

^CVeterinary Services, Animal and Plant Health Inspection Service U.S. Department of Agriculture, Mercer Corporate Park, 320 Corporate Boulevard, Robbinsville, NJ 08691

^DCornell University, College of Veterinary Medicine, Diagnostic Laboratory, Ithaca, NY 14852

Received July 31, 2002

SUMMARY. A multiplex real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) assay for the simultaneous detection of the H5 and H7 avian influenza hemagglutinin (HA) subtypes was developed with hydrolysis type probes labeled with the FAM (H5 probe) and ROX (H7 probe) reporter dyes. The sensitivity of the H5-H7 subtyping assay was determined, using *in vitro* transcribed RNA templates, to have a reproducible detection limit for H7 of approximately 10^4 HA gene copies and approximately 10^4 – 10^5 HA gene copies of H5. A direct comparison of H5-H7 multiplex RRT-PCR with hemagglutination inhibition (HI) was performed with 83 AI RRT-PCR and virus isolation positive tracheal and cloacal swab samples obtained from various avian species and environmental swabs from live-bird markets in New York and New Jersey. Both multiplex RRT-PCR and HI agreed on the subtype determination of 79 (95.2%) of the 83 samples, of which 77 were positive for H7 and two were determined to be non-H5/non-H7 subtypes. No samples were determined to be the H5 subtype by either assay.

RESUMEN. Desarrollo de una prueba múltiple de transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real como herramienta de diagnóstico para influenza aviar.

Se desarrolló una prueba múltiple de transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real para la detección simultánea de los subtipos de hemoaglutinina H5 y H7 de influenza aviar empleando sondas, las cuales al ser hidrolizadas por la acción de la polimerasa ADN emiten fluorescencia, marcadas con las tinciones fluorescentes FAM (sonda H5) y ROX (sonda H7). Se determinó la sensibilidad de la prueba de tipificación H5-H7 empleando moldes de ARN transcritos *in vitro*, las cuales determinaron un límite de detección reproducible de 10^4 copias para el gen de la hemoaglutinina H7 y de aproximadamente 10^4 a 10^5 copias para el gen de la hemoaglutinina H5. Se realizó una comparación directa entre la prueba múltiple H5-H7 de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real y la prueba de inhibición de la hemoagglutination empleando 83 muestras positivas a la prueba múltiple de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real y al aislamiento viral, obtenidas a partir de hisopos traqueales y cloacales de varias especies aviares e hisopos del medio ambiente, obtenidos a partir de centros de mercadeo de aves vivas en Nueva York y Nueva Jersey. Se observó una similitud entre las dos pruebas en la determinación de los subtipos en 79 de las 83 muestras (95.2%), de las cuales, 77 fueron positivas a H7 y dos no correspondieron a los subtipos H5 ni H7. Ninguna de las pruebas detectó muestras del subtipo H5.

Key words: avian influenza, real-time RT-PCR, subtype determination

Abbreviations: AIV = avian influenza virus; bp = base pairs; CAF = chorioallantoic fluid; HA = hemagglutinin; HI = hemagglutination inhibition; PCR = polymerase chain reactions; RRT-PCR = real-time reverse transcriptase-polymerase chain reaction; VI = virus isolation

Real-time reverse transcriptase-polymerase chain reaction (RRT-PCR) has been successfully applied as a rapid alternative to virus isolation (VI) in embryonating eggs for avian influenza virus (AIV) detection and subsequent subtyping for the H5 and H7 hemagglutinin (HA) subtypes (4,5). Continued efforts to improve this assay have included the development of a multiplex assay, which involves performing two or more independent polymerase chain reactions (PCR) simultaneously in a single reaction tube. Multiplex PCR based assays, including standard PCR and real-time PCR assays, have previously been applied to viral diagnostics for the detection of multiple viruses with a single test (5,8) and for the determination of viral subtypes or strains in clinical specimens, including influenza in humans (6). Major advantages of multiplexing include decreased costs and more efficient sample processing.

We have applied multiplex RRT-PCR to the simultaneous detection of the H5 and H7 HA subtypes of AIV. Rapid identification of these subtypes in surveillance and eradication programs is particularly important due to their association with highly pathogenic avian influenza viruses in poultry. Additionally, these subtypes are known to be currently circulating in the live-bird market system in the northeast United States (2,3).

MATERIALS AND METHODS

Multiplex RRT-PCR for H5 and H7 Subtyping. RNA was extracted from clinical samples with the Qiagen RNeasy Kit (Qiagen, Valencia, CA). RRT-PCR reactions were carried out in a 25 μ l volume in the Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). The Qiagen one-step RT-PCR kit (Qiagen) was used in accordance with the manufacturers' instructions with the following changes: $MgCl_2$ was added to a final concentration of 3.75 mM, and 320 μ M of each dNTP was used per reaction. Hydrolysis type probes were used at a final concentration of 0.15 μ M each, and 10 pmol of each primer was used per reaction.

Primer and probe nucleotide sequences for both H5 and H7 were identical to those described for the single reaction assay (4,5). The H7 probe was labeled on the 5' end with the ROX dye and the 3' label was black hole quencher dye (BHQ) 2 (IDT, Coralville, IA). The H5 probe was labeled with FAM at the 5' end and the 3' end label was BHQ 1 (IDT).

The reverse transcription cycling conditions were 30 min at 50°C, then 15 min at 95°C. PCR cycling conditions consisted of 45 cycles of 95°C for 10 sec, 54°C for 30 sec, and 72°C for 10 sec. The fluorescent signal was acquired during the annealing step.

Positive control templates were approximately 80 pg of *in vitro* transcribed H5 or H7 RNA. Positive

controls for each subtype (H5 or H7) were run in separate tubes for maximum sensitivity.

Live-bird market sampling. Samples were obtained from birds in, or from the premises of, live-bird markets in New York and New Jersey during January, February, and March of 2002. Each sample consisted of a pool of up to five tracheal, cloacal, or environmental swabs in brain-heart infusion broth. Swabs were obtained from each avian species present in the market at the time of sampling and from the environment as described previously (4). Briefly, tracheal and cloacal swabs were obtained from five birds of each species and lot present at the time the market was sampled, except ducks, from which only cloacal swabs were obtained. Environmental swabs were taken from the following areas in each market: the office, bird area, slaughter area, and red meat area if present.

Virus isolation (VI) and hemagglutination-inhibition (HI). VI was performed identically as described previously in Spackman *et al.* (4,5). Hemagglutinin inhibition tests with subtype specific antisera were used to subtype all hemagglutination-positive samples. For the hemagglutination-inhibition test (HI), chorioallantoic fluid (CAF) was standardized to four HA units and HA mixed with an equal volume of influenza subtype reference serum at a titer between 1:32 and 1:64. Reference serum and CAF were incubated for 30 min at room temperature; 0.5% chicken RBCs were added and mixed. The assay was evaluated for HI after incubation at room temperature for 30 min.

Only samples that were positive by both assays for AI detection were compared for subtyping to ensure that only samples that were subtyped by both assays were evaluated.

***In vitro* transcribed RNA.** Target genes were cloned into the pAMP1 vector (Life Technologies, Rockville, MD) as previously described (7) and were transcribed with the RiboMax kit (Promega, Madison, WI) from the T7 promoter as per the kit instructions and quantitated by UV spectrophotometer.

RESULTS

Assay detection limits. Detection limits for H5 and H7 in the multiplex assay were determined with *in vitro* transcribed RNA. The reproducible detection limit for H7 was 500 fg of RNA or approximately 10^4 gene copies and for H5 was 500–1000 fg of RNA or approximately 10^4 – 10^5 gene copies. When both H5 and H7 templates were present in a single reaction, the sensitivity for H5 decreased approximately tenfold and the sensitivity for H7 decreased marginally, as opposed to reactions where either the H5 or H7 template was present.

Comparison of H5/H7 multiplex RRT-

PCR with HI. Eighty-three swab samples that were positive for AIV by both RRT-PCR and VI were used to compare HI and multiplex RRT-PCR for subtype determination. The H7 subtype was detected in 77 samples by both multiplex RRT-PCR and HI (Table 1). Four samples were classified as H7 by HI only; multiplex RRT-PCR detected H7 in 77 of 81 (95.1%) samples that were determined to be H7 by HI. Two of the four AIV positive samples that were identified as H7 by HI, but were negative by RRT-PCR for H7, were subsequently tested with the H7 single reaction test as described in Spackman *et al.* (4). One sample was positive.

No samples were determined to contain H5 subtype virus by either assay. Two samples were determined to be neither H5 nor H7 HA subtypes by both assays and were determined to be the H4 subtype by HI.

Overall, multiplex RRT-PCR and HI agreed in subtype determination for 79 of 83 (95.2%) samples.

DISCUSSION

Multiplex PCR assays confer several advantages as compared to a single reaction format. Increased efficiency is one major advantage because results are obtained simultaneously instead of sequentially. Multiplexed PCR reactions can also save money, since although the cost of multiplexed PCR reactions is slightly more than a single reaction because two sets of primers and probes are used, it is substantially less than two separate reactions. Importantly, however, sensitivity may be decreased with multiplexed reactions as compared to a single reaction format. Decreased sensitivity with multiplex PCR is often due to primer-dimer and primer/probe-dimer formation, which subsequently inhibit the reaction (reviewed in 9).

Because smaller products tend to be favored in multiplex PCR reactions and can outcompete larger products for reaction components (1,9), real-time PCR is considered to be a better technique for multiplex PCR than standard PCR, since the amplicons in multiplex real-time PCR reactions can be the same size since sequence specific dye labeled probes are used to identify the specific products instead of size differences. Additionally products that are close in size since will minimize the effects on the efficiency of reverse transcription by competition between amplicons of different sizes (1).

In this case, the primers and probes were chosen

Table 1. Number of RRT-PCR and VI swab samples positive for AIV determined to be the H5, H7, or a non-H5/non-H7 hemagglutinin subtype by multiplex RRT-PCR and hemagglutination inhibition.

HI result	RRT-PCR result	Number of samples
H7	H7	77
H5	H5	0
Non-H5/Non-H7	Non-H5/Non-H7	2
H7	Non-H5/Non-H7	4
Non-H5/Non-H7	H7	0

because they had been previously characterized (4,5) and because of sequence constraints in the templates, although the H5 product is over twice the size of the H7 product (98 base pairs [bp] *vs.* 229 bp for H7 and H5 respectively). The size difference may have affected the relative efficiency of the H5 and H7 tests. The detection limit for H7 in the H5-H7 multiplex RRT-PCR assay was the same as what was observed for a single reaction format based on *in vitro* transcribed RNA detection (when only the H7 template was present). However, there was some loss of sensitivity for H5 detection when multiplexed with H7, even when only the H5 template was present. The sensitivity of both assays was decreased when both H5 and H7 template RNA were present in a single reaction; the sensitivity of H5 was decreased further than that of H7. Inhibition of the H5 assay when multiplexed with the H7 assay is probably due to the larger H5 product.

Based on a direct comparison of HI and multiplex RRT-PCR, with a limited number of clinical samples, detection of H7 was similar to HI. Multiplex RRT-PCR detected H7 in 95.2% of the samples that were determined to be H7 by HI. These results are very close to those observed for the H7 single reaction assay, which detected H7 in 96% of the samples determined to be the H7 subtype by HI (4). Negative results by multiplex RRT-PCR for H7 with samples that were determined to be H7 by HI (false negatives) were probably due to assay sensitivity. Results of the single reaction H7 test with two HI H7 positive, RRT-PCR H7 negative samples support this. Although only one sample was positive when retested, the cycle threshold was very high, indicating a very low amount of initial template. Therefore, this sample probably produced false negative results with the multiplex assay because the amount of starting template was below or near the assay detection limit (notably, it was very close to the detection limit for the single reaction

assay). It also cannot be ruled out that sequence differences between the isolates in the clinical samples and the primer and probe sequences could account for false negatives or decreased assay efficiency.

Although the H7 detection assay performed well, more testing is required to definitively validate this assay. The H5 multiplex RRT-PCR needs to be evaluated with clinical samples and compared to HI; the performance of this test cannot be assessed since there were no H5 positive samples obtained during this study. Because it is rapid (results can be obtained in less than 2 hr) and specific, availability of a multiplex RRT-PCR test for the detection of the H5 and H7 HA subtypes should be a valuable tool for AIV surveillance and detection.

REFERENCES

1. Hayward, A. L., P. J. Oefner, D. B. Kainer, C. A. Hinojos, and P. A. Doris. Kinetics of competitive reverse transcriptase-PCR. In: *PCR applications: protocols for functional genomics*. M. A. Innis, D. H. Gelfand, and J. J. Sninsky, eds. Academic Press, San Diego, CA. pp. 231–261. 1999.
2. Pomeroy, B. Avian influenza. In: *Proc. 98th Annual Meeting of the U.S. Animal Health Association*. U.S. Animal Health Association, Richmond, VA. pp. 512–523. 1994.
3. Senne, D. NVSL Report on avian influenza. In: *Proc. 101st Annual Meeting of the U.S. Animal Health Association*. U.S. Animal Health Association, Richmond, VA. pp. 497–498. 1997.
4. Spackman, E., D. A. Senne, L. L. Bulaga, T. J. Myers, M. L. Perdue, L. P. Garber, K. Lohman, L. T. Daum, and D. L. Suarez. Development of real-time RT-PCR for the detection of avian influenza virus. *Avian Dis.* 47:1079–1082. 2002.
5. Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez. Development of a real-time RT-PCR assay for type A influenza and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* 40:3256–3260. 2002.
6. Stockton, J., J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J. Clin. Microbiol.* 36:2990–2995. 1998.
7. Suarez, D., and M. Perdue. Multiple alignment comparison of the non-structural genes of influenza A viruses. *Virus Res.* 54:59–69. 1998.
8. Vet, J. A. M., A. R. Majithia, S. A. E. Marras, S. Tyyagi, S. Bube, B. J. Pohesz, and F. R. Kramer. Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl. Acad. Sci. U.S.A.* 96:6394–6399. 1999.
9. Zangenberg, G., R. K. Saiki, and R. Reynolds. Multiplex PCR: optimization guidelines. In: *PCR applications: protocols for functional genomics*. M. A. Innis, D. H. Gelfand, and J. J. Sninsky, eds. Academic Press, San Diego, CA. pp. 73–94. 1999.